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(71) Applicant: MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (72) Inventors: CONNOLLY, Thomas, M. ; 410 Calverton Court, Harleysville, PA 19438 (US). KELLER, Paul, M. ; 2057 Spring Valley Road, Lansdale, PA 19446 (US). WAXMAN, Lloyd, H. ; RRI Box 146C Byers Road, Ottsville, PA 18942 (US).		Published <i>With international search report.</i>	

(54) Title: PROTEIN FOR INHIBITING COLLAGEN-STIMULATED PLATELET AGGREGATION

(57) Abstract

A protein which inhibits collagen-stimulated platelet aggregation. The protein has a molecular weight of approximately 17,000. A method of isolating the protein from *Ornithodoros moubata* and using the protein to prevent or delay blood coagulation by blocking the stimulation of platelet aggregation by collagen is also described. The protein is useful in the prevention, prophylaxis, therapy and treatment of thrombotic diseases.

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10 TITLE OF THE INVENTION

PROTEIN FOR INHIBITING COLLAGEN-STIMULATED PLATELET
AGGREGATION

BACKGROUND OF THE INVENTION

15 Normal hemostasis in man is governed by a complex series of interrelated mechanisms involving both cellular and humoral biochemical components. The biochemical pathway involves injury to intact endothelial cells, stimulation of platelets and activation of coagulation mechanisms. When a vessel is damaged and the subendothelium is exposed, platelets very rapidly adhere to some of the constituents of the vessel wall, notably collagen. Although platelets also adhere to other subendothelial 20 constituents, only collagen has been reported to stimulate platelets to release their granule contents and recruit other platelets to the injury site.

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SUMMARY OF THE INVENTION

The invention comprises a protein (Moubatin) isolated from Ornithodoros moubata tick extract which blocks collagen-stimulated platelet aggregation. The 5 protein has a molecular weight of approximately 17,000. The invention also comprises methods of preparing the protein, such as by purifying the protein from Ornithodoros moubata extract, and a method of using the protein to prevent or delay blood 10 coagulation by blocking collagen-stimulated platelet aggregation. The protein is useful in the prevention, prophylaxis, therapy and treatment of thrombotic diseases.

15 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the yeast vector used for expression of the Moubatin synthetic gene sequence.

DETAILED DESCRIPTION OF THE INVENTION

20 The invention encompasses the preferred protein isolated from Ornithodoros moubata or expressed in transformed Saccharomyces cerevisiae, and homologs, isoforms or genetic variants, provided that each one blocks collagen-stimulated platelet 25 aggregation and reacts with antibodies specific for the specified protein.

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Proteins

The invention includes Moubatin, variations on the disclosed purified protein sequence which conserve the activity of the disclosed sequence, including fragments or subunits, naturally occurring mutations, allelic variants, randomly generated artificial mutants and intentional sequence variations which conserve activity. Fragments or subunits refers to any portion of the sequence which contains fewer amino acids than the complete protein, e.g. partial sequences excluding portions at the N- and/or C-termini of the complete protein.

Proteins of the invention also include disclosed recombinant protein sequences which conserve the activity of the purified protein sequences. Also included are hybrid proteins, such as fusion proteins or proteins resulting from the expression of multiple genes within the expression vector, and may include a polypeptide having the specific activity of a disclosed protein linked by peptide bonds to a second polypeptide.

It will be understood that other variants of the proteins of the present invention are included, especially any variants that differ from the isolated protein only by conservative amino acid substitution. Such conservative amino acid substitutions are defined as "sets" in Table I of Taylor, J. Mol. Biol., 188, 233 (1986).

Moubatin is the preferred protein of the invention. Preferably, the protein is obtained by expression in transformed Saccharomyces cerevisiae as described below.

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Identification of Inhibition of
Collagen-Stimulated Platelet Aggregation

Blood was anticoagulated with acid citrate dextrose, and the human platelets were washed and isolated in modified Tyrode's buffer with no Ca⁺⁺, pH 6.5. The final platelet suspension was in a modified Tyrode's buffer without Ca⁺⁺ (134 mM NaCl, 3 mM NaH₂PO₄, 2 mM MgCl₂, 5 mM glucose, 12 mM NaHCO₃, 3.5 mg/ml bovine serum albumin at pH 7.4) adjusted to 3 x 10⁸ platelets/ml after counting in a model ZM Coulter Counter (Hialeah, FL). Aggregation of the washed platelets was used to monitor platelet inhibitory activity in the tick soluble extracts and for each of the fractions tested throughout the purification of Moubatin. The tick extract, column fraction, or aliquot of purified protein was incubated with 0.2 or 0.25 ml platelets at 37°C with 0.2 mg/ml human fibrinogen. Collagen at 1 or 2 µg/ml, or another agonist, was added and the aggregation was monitored in a Sienco or a Chronolog aggregometer. The percentage inhibition was calculated based on the final extent of aggregation of the test samples compared to the appropriate buffer control. Inhibitory activity is defined as a decrease in the rate and/or extent of platelet aggregation.

Identification of Blocking of
Platelet Adhesion to Collagen

Recombinant, yeast-expressed Moubatin does not inhibit the adhesion of washed platelets to Collagen-coated microtiter plates.

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Polystyrene 96-well microtiter plates (Costar, Cambridge, MA) are coated with 100 µl per well of 40 µg/ml collagen dissolved in 5mM acetic acid for 1 hour at room temperature followed by blocking of the non-specific cell binding sites by addition of 200 µl of 10 mg/ml heat-denatured BSA for 1 hour. Control wells are coated with BSA only. The wells are rinsed three times with HEPES buffered saline (HBS) containing 20 mM HEPES, pH 7.4, 0.14 M NaCl, and 2 mM MgCl₂. 100 µl of washed platelets are incubated with various concentrations of Moubatin or buffer as a control for 5 min at room temperature and then added to each collagen coated well and incubated at room temperature for 45 min., nonadherent platelets are removed by aspiration, and the wells are rinsed three times with 200 µl of HBS. The number of adhered platelets is determined by protein assay, using the BCA reagent and measuring the absorbance of each well at 562 nM.

Example 1

Isolation of Collagen-stimulated Platelet Aggregation Inhibitor From
25 Ornithodoros moubata Extract.

Purification

30 Ornithodoros moubata ticks were obtained frozen from Antibody Associates, Ltd. (Bedford, TX) and stored at -70°C until used. A typical preparation was initiated with 400-500 whole ticks

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constituting about 12 grams of material. The ticks
were disrupted in batches of 50 with a Polytron
(Brinkmann Inst. Co., Westbury, NY) in 10 ml of 25 mM
Hepes buffer, pH 7.0, containing 0.15 M NaCl and the
5 protease inhibitors E-64, pepstatin, chymostatin, and
leupeptin (50 uM each). The homogenate was
centrifuged at 100,000 xg for 20 min and the
resulting pellets were reextracted in 5 ml of the
same buffer and recentrifuged. The combined
10 supernatants (approx. 800 mg protein) were tested for
inhibitory activity. Extracts were prepared
similarly for the purification of Moubatin.

The crude soluble extract was diluted 3-fold
with water to lower the ionic strength and applied to
15 a 25 ml Fast Q Column equilibrated in 25 mM Bis-Tris
(pH 7.0). The column was washed with the same buffer
until the absorbance at 280 nm decreased to less than
0.05. The bound proteins were eluted with 0.8 M NaCl
(100 ml) in the same buffer, dialyzed against water
20 to removed the salt, and lyophilized in the presence
of 0.1 mg/ml sucrose. The lyophilized material was
applied to a column of Sephadex G-50 Superfine (2.5 x
50 cm) equilibrated in 50 mM Hepes (pH 7.0).

The fractions that contained platelet
25 aggregation inhibitory activity, which eluted in one
peak, were pooled and applied to an anion exchange
column (Pharmacia Mono Q, 1 x 10 cm) equilibrated in
20 mM Bis-Tris (pH 6.0) controlled by an FPLC
system. The bound proteins were eluted with a
30 gradient of NaCl (0 to 0.3 M) in the same buffer.
Two peaks of inhibitory activity (designated AP-1 and

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AP-2) were found which eluted at approximately 0.12 and 0.25 M NaCl. The active fractions in each were pooled and concentrated by lyophilization. The dried material was redissolved in 1.5 ml H₂O and applied 5 to a column (1.5 x 50 cm) of Sephadex G-75 Superfine equilibrated in 50 mM Hepes (pH 7.0). On gel filtration both AP-1 and AP-2 eluted in one peak with an M_r ~15-20 kDa.

10 The final purification step consisted of fractionation of the pooled activity after gel filtration on repeated reverse phase HPLC on a Pro RPC column (0.5 x 10 cm)(Pharmacia) controlled by an FPLC system. The column was equilibrated in 0.1% (v,v) trifluoroacetic acid in water and the bound 15 proteins were eluted with a gradient of acetonitrile (0 to 50%) also in 0.1% trifluoroacetic acid. 0.5 ml fractions were collected and the solvents were removed by centrifugation under vacuum in a Speed Vac. Fractions were reconstituted with ~100 µl 20 mM Hepes (pH 7.0) before assay and analysis by SDS-PAGE.

20 Moubatin has a molecular weight of 17,256, which is deduced from the predicted amino acid sequence, and the following amino acid composition:

	<u>Amino Acid</u>	<u>Residues/mole</u>
	Ala	10
30	Arg	4
	Asn	6
	Asp	15

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	Cys	6
	Gln	9
	Glu	7
	Gly	13
5	His	2
	Ile	5
	Leu	12
	Lys	16
	Met	2
10	Phe	4
	Pro	5
	Ser	13
	Thr	12
	Trp	3
15	Tyr	5
	Val	7

Example 2

20 In vitro Activity of the Moubatin

Our studies show that while platelet aggregation is stimulated by 2 μ g/ml collagen,
addition of the collagen-stimulated platelet
aggregation inhibitor isolated in Example 1 inhibits
such stimulation. The IC₅₀ for this inhibition was
25 50 nM.

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Moubatin is a specific inhibitor of collagen-induced platelet aggregation. At a concentration that blocks platelet aggregation induced by collagen, moubatin does not inhibit aggregation initiated by other agonists of platelets including thrombin, ADP, arachidonic acid, ristocetin, and A23187. Moubatin also inhibits collagen-induced intracellular Ca^{++} mobilization. Unlike other known inhibitors (e.g. LAPP as described in Journal of Biological Sciences Vol. 267 (1992) (in press)) of collagen stimulation of platelets, moubatin does not block platelet adhesion to collagen in adhesion assays.

Recombinant DNA Technology
Recombinant DNA technology may be used to produce proteins of the invention, preferably Moubatin. This technology allows segments of genetic information, DNA, from different cells, and usually from different organisms, to be joined end-to-end outside the organisms from which the DNA was obtained and to incorporate this hybrid DNA into a cell that will allow the production of the protein for which the original DNA encodes. Genetic information, DNA or mRNA, is isolated and incorporated into an appropriate cloning vector, and transduced into an appropriate host cell.

Cloning vectors useful for this technology include a DNA sequence which accommodates specific experimental foreign DNA. The vectors are introduced into host cells that can exist in a stable manner and express the protein dictated by the experimental DNA. Cloning vectors may include plasmids, bacteriophage, viruses and cosmids.

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Expression vectors are DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. These vectors can express either procaryotic or eucaryotic genes in a variety of cells such as bacteria, yeast, insect and mammalian cells. Proteins may also be expressed in a number of virus systems. A suitably constructed expression vector contains an origin of replication for autonomous replication in host cells, selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. Promoters are DNA sequences that direct RNA polymerase to bind to DNA and initiate RNA synthesis; strong promoters cause such initiation at high frequency. Expression vectors may include, but are not limited to cloning vectors, modified cloning vectors and specifically designed plasmids or viruses.

20

Expression Systems

The preferred expression system for preparing recombinant Moubatin is described in Example 3. The system uses yeast strain Saccharomyces cerevisiae BJ1995.

25 Expression systems useful in the eucaryotic systems of the invention comprise promoters derived from appropriate eucaryotic genes. A class of promoters useful in yeast, for example, include promoters for synthesis of glycolytic enzymes, including those for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. (1980) 255:2073). Other promoters include those from the enolase gene

30

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(Holland, M.J., et al., J. Biol. Chem. (1981) 256:1385) or the Leu2 gene obtained from YEpl3 (Broach, J., et al., Gene (1978) 8:121).

5

Vector Construction

The expression vector preferred for preparing recombinant Moubatin in yeast, is described in Example 3 and Figure 1.

10

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are 15 cleaved, tailored, and religated in the form desired.

20

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which 20 are specified by the manufacturer of these commercially available restriction enzymes. See, e.g. New England Biolabs, Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 µl of 25 buffer solution. Typically, an excess of restriction enzyme is used to ensure complete digestion of the DNA substrate. Incubation times of about 1 to 2 hours at about 37°C are workable, although variations can be tolerated. After each incubation, the 30 digestion product is exposed to phenol/chloroform extraction and may be followed by running over a Sephadex® G-50 spin column. If desired, size separation of the cleaved fragments may be performed

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by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology 5 (1980)65: 499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using 10 incubation times of about 15 to 25 minutes at 20 to 25°C in 50 mM Tris, pH 7.6, 50 mM NaCl, 6mM MgCl₂, 6mM DTT and 5-10 µMdNTPs. The Klenow fragment fills in 5' overhangs but removes protruding 3' single strands, even in the presence of the four dNTPs. If 15 desired, selective repair can be performed by supplying selected dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated followed 20 by running over a Sephadex® G-50 spin column. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

As mentioned above, oligonucleotides may be 25 prepared by the triester method of Matteucci, et al. (J. Am. Chem. Soc. (1981) 103:3185) or using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labelling is achieved using an 30 excess, e.g., approximately 10 units of polynucleotide kinase to 0.1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles ³²P-ATP(2.9 mCi/mmol), 0.1 mM spermidine, 0.1 mM EDTA.

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Ligations are performed in 15-30 μ l volumes under the following standard conditions and temperatures: 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 10 mM DTT, 33 μ g/ml BSA, 10 mM-50 mM NaCl, and 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μ g/ml total DNA concentrations (5-100 nM total end 10 concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 μ M total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na⁺ 15 and Mg²⁺ using about 1 unit of BAP per μ g of vector at 60°C for about 1 hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol 20 precipitated and desalted by application to a Sephadex® G-50 spin column. Alternatively, 25 religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

For portions of vectors derived from cDNA or 30 genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This is conducted using a primer synthetic oligonucleotide complementary to a single stranded

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plasmid or phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is
5 used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar,
10 permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence.
15 The resulting plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with
20 the probe are then picked, cultured, and the DNA recovered.

Probing cDNA Libraries

cDNA or genomic libraries are screened using the colony or plaque hybridization procedure. Each
25 plate containing bacterial colonies (or recombinant phage-infected bacteria) is replicated onto duplicate nitrocellulose filter papers (S & S type BA-85) and, for bacterial colony screens, the colonies are allowed to grow at 37°C for 14-16 hours on L agar
30 containing 50 µg/ml Amp. The bacteria are lysed, plasmid or phage and DNA fixed to the filter by sequential treatment for 5 minutes each with 0.2N NaOH, 1.5M NaCl, then 0.5 M Tris pH 7.5, 1.5M NaCl

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and then 2 x standard saline citrate (2 x SSC).
Filters are air dried and baked at 80°C for 2 hours.
The duplicate filters are prehybridized at 42°C for
5 6-8 hours with 10 ml per filter of DNA hybridization
buffer (5x SSC, pH 7.0, 5x Denhardt's solution
(polyvinyl pyrrolidine, plus Ficoll and bovine serum
albumin; 1 x = 0.02% of each), 50 mM sodium phosphate
buffer at pH 7.0, 0.2% SDS, 20 µg/ml polyU, and 50
10 µg/ml denatured salmon sperm DNA.

The samples are hybridized with kinased
probe under conditions which depend on the stringency
desired. Typical moderately stringent conditions
employ a temperature of 42°C for 24-36 hours with 1-5
15 ml/filter of DNA hybridization buffer containing
probe. For higher stringencies, high temperatures
and shorter times are employed. The filters are
washed four times for 30 minutes each time at 37°C
with 2 x SSC, 0.2% SDS and 50 mM sodium phosphate
20 buffer at pH 7, then are washed twice with 2 x SSC
and 0.2% SDS, air dried and are autoradiographed at
-70°C for 2 to 3 days.

Oligonucleotide Primers

25 Oligonucleotide primers are prepared which
will hybridize to different strands of the desired
sequence and at relative positions along the sequence
such that an extension product synthesized from one
primer, when it is separated from its template
30 (complement), can serve as a template for extension
of the other primer into a nucleic acid of defined
length. The primers may be prepared using any suit-
able method, such as, for example, the phosphotri-

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ester and phosphodiester methods, described respectively in Narang, S.A., et al. Meth. Enzymol., 68, 90 (1979) and Brown, E.L. et al., Meth. Enzymol., 68, 109 (1979), or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., Tetrahedron Letters (1981), 22: 1859-1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

15

Polymerase Chain Reaction Amplification

Large amounts of DNA coding for the protein may be obtained using polymerase chain reaction (PCR) amplification techniques as described in Mullis et al., U.S. Patent No. 4,800,159. The extension product of one primer, when hybridized to another primer, becomes a template for the production of the nucleic acid sequence.

25

The primer template complexes act as substrate for DNA polymerase which, in performing its replication function, extends the primers. The region in common with both primer extensions, upon denaturation, serves as template for a repeated primer extension.

30

Tag DNA Polymerase catalyzes primer extension in the amplification process. The enzyme is a thermostable DNA polymerase isolated from Thermus aquaticus. Because it stays active through repeated elevations to high denaturation temperatures, it needs to be added only once. Deoxynucleotide

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triphosphates provide the building blocks for primer extension.

The nucleic acid sequence strands are heated until they separate, in the presence of oligonucleotide primers that bind to their complementary strand at a particular site of the template. This process is continued with a series of heating and cooling cycles, heating to separate strands, and cooling to reanneal and extend the sequences. More and more copies of the strands are generated as the cycle is repeated. Through amplification, the coding domain and any additional primer-encoded information such as restriction sites or translation signals (signal sequences, start codons and/or stop codons) is obtained. PCR protocols are often performed at the 100 µL scale in 0.5-mL microcentrifuge tubes. The PCR sample may be single- or double-stranded DNA or RNA. If the starting material is RNA, reverse transcriptase is used to prepare first strand cDNA prior to PCR. Typically, nanogram amounts of cloned template, up to microgram amounts of genomic DNA, or 20,000 target copies are chosen to start optimization trials.

PCR primers are oligonucleotides, typically 15 to 30 bases long, and are complementary to sequences defining the 5' ends of the complementary template strands. Non-template complementary 5' extensions may be added to primers to allow a variety of useful post amplification operations on the PCR product without significant perturbation of the amplification itself. It is important that the two

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PCR primers not contain more than two bases complementary with each other, especially at their 3' ends. Internal secondary structure should be avoided
5 in primers.

Because Taq DNA Polymerase has activity in the 37-55°C range, primer extension will occur during the annealing step and the hybrid will be stabilized. The concentrations of the primers are preferably equal
10 in conventional PCR and, typically, within 0.1-to 1- μ M range.

In the standard PCR protocol, each deoxynucleotide triphosphate concentration is preferably about 200 μ M. The four dNTP
15 concentrations are preferably above the estimated K_m of each dNTP (10-15 μ M).

Preferably, PCR buffer is composed of about 500 mM potassium chloride, 100 mM Tris-HCl (pH 8.3 at room temperature), 15 mM magnesium chloride, and
20 0.01% w/v gelatin. In the presence of 0.8 mM total dNTP concentration, a titration series in small increments over the 1.5-to 4-mM range will locate the magnesium concentration producing the highest yield of a specific product. Too little free magnesium
25 will result in no PCR product and too much free magnesium may produce a variety of unwanted products.

Preferably, in a 100- μ L reaction volume, 2.0 to 2.5 units of Taq DNA Polymerase are recommended. The enzyme can be added conveniently to
30 a fresh master mix prepared for a number of reactions, thereby avoiding accuracy problems associated with adding individual 0.5- μ L enzyme

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aliquots to each tube. A typical PCR protocol for amplification of the DNA template includes a 1 minute 94°C denaturation step, a 1 minute 37°C primer 5 annealing step, and a 2 minute 72°C primer extension step. This will amplify a 500 base-pair product at least 100,000-fold in 25 cycles.

During DNA denaturation, sufficient time must be allowed for thermal equilibration of the 10 sample. The practical range of effective denaturation temperatures for most samples is 92-95°C, with 94°C being the standard choice.

Primer annealing is usually performed first at 37°C, and the specificity of the product is 15 evaluated. If unwanted bands are observed, the annealing temperature should be raised in subsequent optimization runs. While the primer annealing temperature range is often 37-55°C, it may be raised as high as the extension temperature in some cases. 20 Merging of the primer annealing and primer extension steps results in a two-step PCR process.

Primer extension, in most applications, occurs effectively at a temperature of 72°C and seldom needs optimization. In the two-temperature 25 PCR process the temperature range may be 65-70°C. In situations where enzyme concentration limits amplification in late cycles, the extension is preferably increased linearly with cyclic number. Usually, 25 to 45 cycles are required for extensive 30 amplification (i.e., 1,000,000 fold) of a specific target.

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Transformation

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., Proc. Natl. Acad. Sci. USA (1972) 69:2110, or the RbCl method described in Maniatis et al., Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, p.254 is used for prokaryotes or other cells which contain substantial cell wall barriers. Infection with Agrobacterium tumefaciens (Shaw, C.H., et al., Gene (1983) 23:315) is used for certain plant cells. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546 is preferred. Transformations into yeast are carried out according to the method of Van Solingen, P., et al., J. Bacter. (1977) 130:946 and Hsiao, C.L. et al., Proc. Natl. Acad. Sci. USA (1979) 76:3829.

Example 3 describes a preferred yeast expression system for preparing recombinant Moubatin.

Example 3

Identification of Nucleic Acid Sequence (SEQUENCE ID NO. 1) and Entire Amino Acid Sequence and Expression of 156 Amino Acid Moubatin (SEQUENCE ID NO. 2)

30 Identification of Nucleic Acid Sequence

A lambda ZAP II (Stratagene) tick cDNA library was constructed following the manufacturer's instructions using total tick poly (A) + RNA.

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Total phage DNA from this was isolated using standard DNA isolation procedures. Using standard PCR protocols, the DNA was screened for the presence 5 of moubatin sequences by PCR amplification. PCR analysis was run using primer pairs consisting of a λ arm primer; (primer λ 1:5'-
GCTCT AGA ACT AGT GGA TCC-3') (Sequence ID No. 5), and degenerate oligonucleotide primers whose 10 sequences were based on two Lys-C peptide fragments. The peptide sequence and the corresponding oligonucleotides were the following: YQQWQING (Sequence ID No. 6) which is found within Lys-C fragment L7. The corresponding coding and 15 complementary oligonucleotides were also made. The second Lys-C fragment peptide (L1) DIQCCDEK (Sequence ID No. 7) and the corresponding coding and complementary oligonucleotides were also made. In addition, four of the oligonucleotides based on 20 the two Lys-C peptide fragments had sequences corresponding to a Not 1 restriction endonuclease site built at their 5' end.

These peptides were chosen from the eight available peptide sequences because of their low 25 degeneracy. Intial PCR analysis was performed using 10 ng of total library DNA as a substrate. Each arm primer was used with each of the four degenerate primers to generate product. As a control for non-specific product formation every primer was also 30 run alone with the total library DNA as a substrate.

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Sizes of the PCR products were estimated by comparing migration through a 1.5% agarose gel relative to a ϕ X 174 RF DNA/HaeIII marker (BRL).

5 The product of primer pair λ 1, and the complementary oligonucleotide to LysC fragment (L1) (Sequence ID No. 7) was cut with Not 1 and Spe 1, subcloned into a vector containing Not 1 and Spe 1 sites, and sequenced. The sequencing confirmed that
10 this PCR product contained within it both Lys-C fragments used to construct the primers as well as the Lys-C peptide sequences of fragments L4, L5 and L6.

15 The PCR product of primer pair 1,4 was then used as a hybridization probe to screen the cDNA library directly. This was done to eliminate any possible PCR generated sequence errors, rather than generating the entire cDNA directly using PCR. Screening initially produced numerous positive phage plaques. 20 plaques were isolated and PCR analysis was performed on these 20 positive plaques using primer λ 1 and 4, thus amplifying the 5' end of the cDNA. Five of the 20 plaques gave a product of ~500 base pairs, the remainder gave smaller or no bands. Presumably the absence of a band was due to inserts which were oriented in the opposite direction.

20 Two of the phagemids containing the longest inserts were grown and sequenced. Sequencing revealed an uninterrupted open reading frame of 513 nucleotides which contained all of the Lys-C
25
30

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fragments. It was followed by a 60 nucleotide 3' untranslated region followed by a poly A tract. A hexa nucleotide AATAAA consensus sequence was located
5 15 nucleotides before the poly (A)⁺ tract. Only 12 nucleotides of 5'-untranslated region were found in any of the sequenced clones. This is possibly due to premature termination of the reverse transcriptase during the construction of the tick library.

10 Using the computer program SIGSEQ2, which can be used to predict the region of the protein which is the signal peptide, the predicted peptidase cleavage occurs between amino acids 15 and 16(Ser and Ile). This cleavage results in a mature protein of
15 156 amino acids with a predicted molecular weight of 17,256. The predicted moubatin protein sequence was compared with that of other protein sequences in the Swiss PROT data base. No significant homology was found.

20

Construction of pKH4 α 2

Construction of pKH4 is described in Schultz, et al., Gene 61 (1987) 123-133, which is incorporated by reference. The plasmid pJC197
25 (Schultz et al. Gene 54 (1987) 113-123) is an E. coli - S. cerevisiae shuttle vector which contains a unique BamHI cloning site between the yeast MFaI pre-pro leader and transcriptional terminator, originally described in Kurjan and Herskowitz (1982)
30 ibid. pJC197 was digested with EcoRI + PstI, and the 0.7-kb PstI-EcoRI fragment containing a portion of the MFaI pre-pro-leader, a three-frame translational terminator, and MFaI transcriptional terminator, was gel-purified. GAL10p was isolated

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from YEP51 by digestion with Sau3A, flush-ending with PoIk, ligating with octameric BamHI linkers, and digestion with SalI.

5 The resulting 0.5-kb BamHI-SalI fragment bearing GAL10p was gel purified and ligated to a
10 35-bp SalI-PstI synthetic oligodeoxynucleotide adapter encoding the first 11 bp of the MF α 1 nontranslated leader plus the ATG and first 8 aa of the MF α 1 pre-pro-leader. The resulting 0.5-kb
15 fragment was digested with BamHI, gel-purified, and ligated together with the aforementioned 0.7-kb PstI-EcoRI fragment plus the 4.0kb EcoRI-BamHI vector fragment derived from pBR322. The resulting plasmid,
20 pKH207-1, contains GAL10p fused to the MF α 1 pre-pro-leader plus BamHI cloning site, translational termination codons, and MF α 1 transcriptional terminator. Upon digestion with EcoRI and partial
25 digestion with BamHI, an expression cassette of GAL10p fused to the yeast MF α 1 pre-pro-leader, a unique BamHI cloning site, translational termination codons in all three reading frames, and MF α 1 transcriptional terminator sequence was inserted into the yeast shuttle vector pC1/1 (Rosenberg et al. Nature 312 (1984) 77-80) which contains the yeast 2 μ DNA sequence for stable propagation of the plasmid in yeast at high copy number, to form pKH4.

30 A 213-bp BamHI-PstI fragment encoding aa 9-79 of the ppL was prepared from the plasmid p α 2 (Bayne et al., Gene 66 (1988) 235-244). The plasmid p α 2 contains a portion of the yeast MF α 1 pre-pro sequence (79aa) modified at codons 80 and 81 to create a BamHI site 6 aa upstream from the KEX2 processing site. The region corresponding to codon 9

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(PstI) of the ppL to the BamHI site of pKH4 was removed from pKH4 after digestion with BamHI followed by partial digestion with PstI. The 5 modified vector, pKH4α2 was prepared by replacement of this excised sequence with the BamHI-PstI fragment from pα2. Plasmid pKH4α2 contains the yeast GAL10 promoter, a portion of the MFα1 pre-pro leader (79 aa), a three-frame translational 10 terminator and MFα1 transcriptional terminator, the yeast LEU2 gene, yeast 2μ sequences, pBR322 derived sequences, including the Ap^R gene and the origin of DNA replication (ori).

15 Construction of Moubatin Expression Vector

The predicted mature protein coding sequence was expressed in yeast using the expression vector pKH4α2. The coding sequence was isolated by PCR, generating a DNA fragment using the Bluescript SK 20 phagemid vector containing the moubatin cDNAs as a substrate and the oligo primers, Sequence ID Nos. 3 and 4.

25 5' ATATGGATCC TGTCTTGGA TAAAAGAATT GCATATGCTC
 AAAGCGGA 3'

5' ATATCGGGAT CCTATTTGC GGAAGTCTCG CA 3'

These primers contain a BamH1 site for 30 subcloning and insert codons for a yeast KEX2 protease cleavage site (Lys-Arg) N-terminal to the predicted signal peptidase cleavage site of the native protein. KEX2 protease cleaves on the carboxy

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terminal side of Lys-Arg enabling generation of the authentic N-terminus of moubatin which is produced and secreted by the yeast.

5 The PCR resulted in a blunt end fragment which was digested with BamH1. The correct fragment was obtained after electrophoresis in a 1% agarose gel, excision of the band and electroelution (before ligation the vector was digested with BamH1 and treated with calf intestine alkaline phosphatase).
10 The fragment was purified and then ligated into the yeast expression vector pKH4α2. The sequence and orientation of the final isolated clones was confirmed by DNA sequence analysis.

15 Figure 1 shows construction of the yeast plasmid expression vector used for the expression of the Moubatin gene sequence. Representations are:

20 Moubatin coding sequence

α-mating factor gene sequences

2μ circle sequences

25 pBR322 sequences

leu2^d gene sequences

30 GAL 10 promoter sequence

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Transformation of *Saccharomyces cerevisiae* BJ1995

The haploid *Saccharomyces cerevisiae* strain BJ1995 (MAT, leu², trp¹, prb¹⁻¹¹²², pep⁴⁻³)
5 cir+; a gift from Elizabeth Jones, Carnegie-Mellon University) was made cir° by standard techniques and transformed using the procedure of Hinnen et al. (1978) Proc. Natl. Acad. Sci. USA 75: 1929-1933.

Transformant clones were selected on leucine-minus agar plates and propagated at 30°C in 5 x Leu- medium, containing 4% glucose. At a cell density of A₆₀₀= 6-9, the cells were harvested by centrifugation and resuspended at the same cell density in 5 x Leu- medium containing 2% galactose
10 for induction. After 48 hour induction at 30°C, the cells were removed by centrifugation and the medium was evaluated for the expression of moubatin (see below). A clonal isolate for a pre-Master seed was stored at -70°C in the presence of 17% (v/v) glycerol
15 for all further fermentation and protein purification studies.
20

Purification of Recombinant Moubatin

Yeast cells were sedimented by centrifugation and the remaining cell free culture medium was diluted 5-fold with 20 mM NaAcetate buffer, pH 4.0, and applied to a Fast S sepharose column that had been previously equilibrated with the same buffer. The column was washed with 5 column volumes of the
25 same buffer and the recombinant moubatin eluted with a 0- 1.0 M NaCl gradient in the same buffer. The fractions were screened for moubatin by assay for platelet aggregation inhibitory activity. The peak fractions were pooled, concentrated in a speed vac,
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and applied to a reverse phase C₁₈ HPLC column. Purified recombinant moubatin was eluted with a linear gradient of 17-50% isopropanol, 0.08% 5 trifluoroacetic acid over 45 min. The fractions were dried in a speed vac and dissolved in 20 mM NaAcetate pH 7.3 and assayed for aggregation inhibitory activity and purity by SDS-PAGE. The final isolated moubatin was quantified by amino acid composition 10 analysis and its identity and purity confirmed by amino terminal amino acid sequence analysis.

Deposit

The transformed yeast strain 15 pKH4α2-Moubatin, deposited with the American Type Culture Collection, Rockville, MD, USA, is designated ATCC _____. The deposit was made February 24, 1992 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of 20 Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the 25 Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the 30 invention in contravention rights granted under the authority of any government in accordance with its patent laws.

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Therapy

The proteinaceous substance of this invention, which blocks stimulation of platelet aggregation by collagen, forms pharmaceutically acceptable salts with any non-toxic, organic or inorganic acid. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulphuric and phosphoric acid and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids which form suitable salts include the mono, di and tricarboxylic acids. Illustrative of such acids are, for example, acetic, glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic, 2-phenoxybenzoic and sulfonic acids such as methane sulfonic acid and 2-hydroxyethane sulfonic acid. Salts of the carboxy terminal amino acid moiety include the non-toxic carboxylic acid salts formed with any suitable inorganic or organic bases. Illustratively, these salts include those of alkali metals, as for example, sodium and potassium; alkaline earth metals, such as calcium and magnesium; light metals of Group IIIA including aluminium; and organic primary, secondary and tertiary amines, as for example, trialkylamines, including triethylamine, procaine, dibenzylamine, 1-ethenamine; N,N'-dibenzylethylenediamine, dihydroabietylamine, N-(lower)alkylpiperidine, and any other suitable amine.

The antithrombotic blood concentration of

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the proteinaceous substance of this invention which blocks stimulation of platelet aggregation by collagen is about 200 nMolar (or 3.2 µg/ml).

5 Anticoagulant therapy is indicated for the treatment and prevention of a variety of thrombotic conditions, particularly coronary artery and cerebrovascular disease. Those experienced in this field are readily aware of the circumstances requiring anticoagulant therapy. The term "patient" 10 used herein is taken to mean mammals such as primates, including humans, sheep, horses, cattle, pigs, dogs, cats, rats, and mice.

15 Although the proteinaceous substance of this invention may survive passage through the gut following oral administration, applicants prefer non-oral administration, for example, subcutaneous, intravenous, intramuscular or intraperitoneal; administration by depot injection; or by implant preparation.

20 For parenteral administration the proteinaceous substance of this invention may be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as water and oils with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants.

25 Illustrative of oils which can be employed in these preparations are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general,

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water, saline, aqueous dextrose and related sugar
solutions, ethanol and glycols such as propylene
glycol or polyethylene glycol are preferred liquid
5 carriers, particularly for injectable solutions.

The proteinaceous substance of this invention
can be administered in the form of a depot injection
or implant preparation which may be formulated in such
a manner as to permit a sustained release of the
10 active ingredient. The active ingredient can be
compressed into pellets or small cylinders and
implanted subcutaneously or intramuscularly as depot
injections or implants. Implants may employ inert
15 materials such as biodegradable polymers or synthetic
silicones, for example, Silastic, silicone rubber or
other polymers manufactured by the Dow-Corning
Corporation.

20

25

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT: Waxman, Lloyd
Connolly, Thomas M.
Keller, Paul

(ii) TITLE OF INVENTION: Protein for Inhibiting
Collagen-Stimulated Platelet Aggregation

10 (iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Merck & Co., Inc.
(B) STREET: P.O. Box 2000
(C) CITY: Rahway
(D) STATE: New Jersey
(E) COUNTRY: USA
15 (F) ZIP: 07065

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Parr, Richard S.
25 (B) REGISTRATION NUMBER: 32,586
(C) REFERENCE/DOCKET NUMBER: 18415

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (908) 594-4958
(B) TELEFAX: (908) 594-4720
(C) TELEX: 138825

30

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(2) INFORMATION FOR SEQ ID NO:1:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 595 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

10 (ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAGCAGTCA ACATGATGCT GGTTTGACG ACCCTGATT TCTCCTTTTC TGCGAGCATT	60
GCATATGCTC AAAGCGGATG CTCTGTAAGC GACCCTCTCG ACGCCTTGAA AGCTTCAAG	120
GACGGTGAGA GAACATTCCCT TCTACAGAAA TCCACAGATC CCCAAGCAAG GGACTGCTTG	180
15 AAAGGGACCC CGAACATGGAAA TAGAGACGGC AACACGTTGC CAGTGACAAT GACGTACAAG	240
GACGACTCCA AGTGGGTTTC ATTGAATTGG ATGTTTACTT TGGAAGGCAGC AAATATAGTC	300
GCAACCTTAG AAGGAAAGCG AAAGCAACGG GGGGAACTAG TCTACGACGT GCAAAGTCAT	360
20 GACTGCCATA TTACCAAGCT CTCCCTGGGC GTGTATCAGC AGTGGCAGAG CAATGGCTCA	420
GCAGATGACA AGGACATTAAGTGTGCGAT GAGAAATTAA AGGAGTTGAC GTCTGGTATT	480
GACTACACAA AGCCCCAAGA GAAGGGCTGC GAGACTTCCG CAAAATGAAG AACCTACAGG	540
CATCTCATCA CACTGAAAGA ATTCCAATA AACTGCTTTA TAAACAAAAA AAAAA	595

25 (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 171 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

30 (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Met Leu Val Leu Thr Thr Leu Ile Phe Ser Phe Ser Ala Ser Ile
1 5 10 15

5 Ala Tyr Ala Gln Ser Gly Cys Ser Val Ser Asp Pro Leu Asp Ala Leu
20 25 30

Lys Ala Phe Lys Asp Gly Ala Gly Thr Phe Leu Leu Gln Lys Ser Thr
35 40 45

Asp Pro Gln Ala Arg Asp Cys Leu Lys Gly Thr Pro Asn Gly Asn Arg
10 50 55 60

Asp Gly Asn Thr Leu Pro Val Thr Met Thr Tyr Lys Asp Asp Ser Lys
65 70 75 80

Trp Val Ser Leu Asn Trp Met Phe Thr Leu Glu Gly Ala Asn Ile Val
85 90 95

15 Ala Thr Leu Glu Gly Lys Arg Lys Gln Arg Gly Glu Leu Val Tyr Asp
100 105 110

Val Gln Ser His Asp Cys His Ile Thr Lys Leu Ser Ser Gly Val Tyr
115 120 125

Gln Gln Trp Gln Ser Asn Gly Ser Ala Asp Asp Lys Asp Ile Lys Cys
20 130 135 140

Cys Asp Glu Lys Phe Lys Glu Leu Thr Ser Gly Ile Asp Tyr Thr Lys
145 150 155 160

Pro Gln Glu Lys Gly Cys Glu Thr Ser Ala Lys
165 170

25 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

30 (ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATATGGATCC TGTCTTGGA TAAAAGAATT GCATATGCTC AAAGCGGA

48

5 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

10 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATCGGGAT CCTATTTGC GGAAGTCTCG CA

32

15 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

20 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTCTAGAAC TAGTGGATCC

20

25 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

30 (ii) MOLECULE TYPE: peptide

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Gln Gln Trp Gln Ile Asn Gly
1 5

10 (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

15 (ii) MOLECULE TYPE: peptide

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp Ile Gln Cys Cys Asp Glu Lys
1 5

25

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WHAT IS CLAIMED IS:

1. A biochemically pure protein having a
5 molecular weight of about 17,000 and a capacity to
inhibit collagen-stimulated platelet aggregation and
which does not inhibit platelet adhesion to collagen,
wherein the protein is derived from the tick
Ornithodoros moubata.

10 2. A protein having the amino sequence
identified as SEQ. ID NO:2 or conservative amino acid
substitutions thereof, or fragments thereof which
inhibit collagen-stimulated platelet aggregation.

15 3. A process for the production of a protein
derived from Ornithodoros moubata tick extract and
having a capacity to inhibit collagen-stimulated
platelet aggregation, comprising the steps of:

20 a) disrupting Ornithodoros moubata
ticks;

25 b) homogenizing and solubilizing tick
extract in an aqueous solution comprising a
buffered salt having a suitable fixed pH to
produce a homogenate;

30 c) centrifuging the homogenate to
produce supernatant protein suspension
fractions; and

d) assaying the fractions and
selecting a product fraction characterized
by its inhibition of collagen-stimulated
platelet aggregation.

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4. The product of Claim 3 wherein the product fraction inhibits collagen-stimulated platelet aggregation and does not inhibit platelet adhesion to collagen.

5
10 5. A therapeutic composition for inhibiting collagen-stimulated platelet aggregation comprising an effective amount of a protein of Claim 2.

15 6. A method of treating a mammal for the prevention of collagen-stimulated platelet aggregation comprising administering to the mammal, in a therapeutically effective dose, a composition of Claim 5.

20 7. A gene having a DNA sequence of SEQ. ID NO:1.

25 8. A recombinant molecule comprising DNA coding for the protein of Claim 1 comprising a DNA sequence coding for expression in a host for the polypeptide of Claim 2 which inhibits collagen-stimulated platelet aggregation and which does not inhibit platelet adhesion to collagen, the DNA sequence being operatively linked to an expression control sequence in the DNA molecule.

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9. A method for producing the polypeptide
of Claim 2 which inhibits collagen-stimulated
platelet aggregation and which does not inhibit
5 platelet adhesion to collagen comprising the steps of:
a) culturing a transformed
Saccharomyces cerevisiae host with a recombinant DNA
molecule, said molecule comprising a DNA sequence
coding for expression of a polypeptide of Claim 2.
10 which inhibits collagen-stimulated platelet
aggregation, the DNA sequence being operatively
linked to an expression control sequence in the DNA
molecule; and
15 b) collecting and isolating the
polypeptide.

20

25

30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/01717

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :435/240.2, 255, 256, 69.1, 320.1; 530/350, 351, 355, 356; 536/27.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 255, 256, 69.1, 320.1; 530/350, 351, 355, 356; 536/27.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	SCIENCE, VOLUME 248, ISSUED 04 MAY 1990, L. WAXMAN ET AL., "TICK ANTICOAGULANT PEPTIDE (TAP) IS A NOVEL INHIBITOR OF BLOOD COAGULATION FACTOR XA", PAGES 593-596, ESPECIALLY PAGE 595.	<u>1-2,4-5</u> <u>3,6-9</u>
X Y	THE JOURNAL OF CELL BIOLOGY, VOLUME 115, NO. 3, PART 2, ISSUED NOVEMBER 1991, J. KARCZEWSKI ET AL., "IDENTIFICATION AND PURIFICATION OF AN INHIBITOR OF CELLULAR ADHESION TO COLLAGEN FROM THE TICK ORNITHODOROS MOUBATA", PAGE 442 A, SEE THE ABSTRACT.	<u>1-2,4-5</u> <u>3,6-9</u>

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
05 APRIL 1993Date of mailing of the international search report
13 APR 1993Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

GIAN WANG



Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01717

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C12N 5/00, 15/00, 1/16, 1/18; C12P 21/06; C07H 15/12; C09H 1/00; C07K 3/00, 13/00, 15/00, 17/00; A61K 37/12.

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